BACILLUS PERFRINGENS: TOXIN AND ANTITOXIN PRODUCTION

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With few exceptions the reports on the bacteriology and immurology of gas gangrene have given only few details regarding the methods found most advantageous for the production of toxin and antitoxin. Therefore it seemed worth while to record certain observations made in the production and standardization of an antiserum to B. perfringens and a combined antiserum to B. perfringens and B. tetani.

CULTURAL METHODS

The detailed experiments of Bull and Pritchett[1] on the factors influencing the artificial production of toxin showed clearly, among other features, the effect of the incubation period, the addition of fresh muscle and of glucose. At first I found the addition of fresh muscle to be sufficiently troublesome to induce one to determine whether it was worth the time necessary, in view of the purpose of the work. It had appeared early in the work that toxin production was closely proportional to infectivity of the culture. Subsequent work appeared to support this conclusion although definite experiments were not planned to determine the exact relationship between these two phenomena. However, they were sufficiently closely related, and the difficulty of maintaining the virulence of the cultures sufficiently marked to make it advisable to investigate any methods whereby the infectivity of the strain might more easily be maintained.

Fresh muscle glucose (0.2%) broth and autoclaved muscle glucose (0.2%) broth cultures were contrasted regarding their infectivity. Six pigeons varying in weight from 300 to 340 gm. were used; 3 of these received fresh muscle broth culture intramuscularly in the following doses, 0.1 c c, 0.5 c c and 0.01 c c; the remaining 3 received the autoclaved muscle culture in the following doses: 0.05 c c, 0.01 c c and 0.005 c c. These cultures were approximately 20 hours old, had been seeded with equal amounts from the same parent culture, and the inoculations were given at 4 p. m. By next morning the 3 pigeons that had received the fresh muscle culture were dead, while the remaining 3 birds all survived, though they showed varying degrees of focal lesion according to the size of the dose.

This experiment had been planned to show whether the effect of adding fresh muscle to the mediums was sufficiently important to make its adoption as a routine measure advisable, and also to insure a successful passage, as at that time the virulence of the culture used had dropped to a low point. This result was so much more favorable for the fresh muscle medium that its use was adopted as a routine measure.

[1] J. Exper. Med. 1917, 26, p. 119, 603, and 867.

This is the technic finally adopted for the preparation of fresh muscle broth:

A healthy rabbit was chloroformed, the back and thigh shaved, and the skin thus prepared scarred with a red hot iron. Knives, long handled scissors, and long forceps were dry sterilized. The back muscles, one side at a time, exposed and appropriate sized pieces of muscle cut and deposited in wide mouthed bottles containing sterile salt solution. It was only with considerable difficulty that freshly cut pieces of muscle could be inserted into even wide culture tubes, because they tend to stick to the sides of the tubes wherever they touch the glass. This difficulty was found to be almost entirely overcome by the use of the salt solution. Throughout the operation the free bunsen flame was lightly and occasionally used on the exposed muscles and for the instruments. The charred pieces of muscle which became attached to the instruments were detached by wiping with small moist pads of sterile cotton wool. When a sufficient number of pieces had been deposited in the bottles of salt solution they were transferred piece by piece into the tubes or flasks of medium, the bottles and medium containers being slightly slanted to allow the use of the free bunsen flame as desired. The time required after the rabbit was shaved to complete the preparation of the medium was about an hour. Incubation for 48 hours for sterility showed that usually less than 1% of the tubes would be contaminated although on two or three occasions about 40% were not sterile. The percentage of contamination for Erlenmeyer flasks of broth was always two or three times that of the tubes.

To prevent evaporation it was found advisable to add sterile paraffin oil to all but a few tubes which were kept for early use, and to incubate again for sterility. The paraffin oil was sterilized in a flask so equipped that the oil could be syphoned off.

Pasteur pipets were used for transplantation from the oil covered cultures, not only because in this way the oil could be avoided and films made, but also because large amounts could be carried over.

The anaerobic method found most suitable was the simple one used by Bull and Pritchett. The oil covered tubes or flasks, after being inoculated, were enclosed in a vacuum jar from which the oxygen was then exhausted. The additional use of pyrogallic acid and KOH was not found to have any advantage.

Contamination, which was liable to appear in the cultures made from the pigeons or guinea-pigs used as a means of maintaining virulence, was controlled by seeding on a slope agar and incubating aerobically. This, and films from the broth culture, proved to be a satisfactory routine method for the control of this factor. The need for the early detection of contamination arose from the difficulty of maintaining the virulence throughout the procedures necessary to obtain again the B. perfringens in pure culture when once contaminated. Rather than resort to this it was found that time was saved by carrying on from an older culture. If a contaminated culture had to be used, frequent subculturing (after 6-8 hours' growth anaerobically) seemed to be the most satisfactory method of again obtaining the bacillus in pure culture without the loss of virulence that invariably accompanied any method involving the use of solid medium.

Bull and Prichett have commented on the aggressin like action of B. perfringens in that the pathogenicity of other micro-organisms is increased when present in the host experimentally infected with B. perfringens. This appeared to play a rôle in my hands on several

occasions. Such a phenomenon opens up several interesting paths that might prove of value in the investigation of other infections, particularly those of the subacute varieties. The observations on the bacterial flora found in association with B. perfringens in war wounds when the toxemia of the patient was marked, although cultures of B. perfringens showed but slight pathogenicity, may well be due, to a great extent, to this characteristic.

Maintenance of Virulence

As already commented on, there was every indication that the potency of the filtrate (toxin) was proportional to the infectiousness of the culture. Unless the utmost care was used great loss in the virulence of the strain would occasionally show itself in the more or less routine fashion in which pigeons were used to maintain this, and considerable loss of time was occasioned in raising the virulence. Under the most careful technic contamination would appear in the cultures made from animal or bird passage. This apparently occurred more readily when guinea-pigs were used instead of pigeons.

On one of these occasions, with the hope of more rapidly increasing virulence, bird to bird passage was tried under the following circumstances: Culture 617 D had lost virulence to such an extent that the inoculation of 0.1 c c of an 18-hour, fresh muscle glucose-broth culture into the breast muscles of a 310 gm. pigeon showed after 24 hours no general intoxication but considerable local swelling and gas infiltration. The pigeon which apparently was likely to recover was chloroformed, the lesion massaged and bruised so that it was possible to withdraw aseptically by means of a syringe and wide bore needle 0.8 c c of thick blood stained semifluid débris. Films made from this showed broken down muscle tissue, blood cells, débris and an average of from 20-40 clearly outlined bacilli per field. With this material two pigeons were inoculated: One pigeon (310 gm.) received the entire 0.8 c c, and the other (290 gm.) the washings obtained by rinsing the syringe with 0.5 c c sterile salt solution. The pigeon which had received the undiluted dose showed a barely perceptible local and no general reaction, while the pigeon that received the diluted material showed no effects. The virulence of this strain was, however, increased by inoculating birds with massive doses of subcultures originating from this same pigeon.

Every attempt to raise the virulence by direct passage methods failed although the virulence was on each of these occasions increased by cultural methods.

I do not mean to suggest that virulence, under appropriate and favorable circumstances, can not be maintained or even increased by direct passage from bird to bird; but the fact that direct passage can fail in these respects provides, I think, a further suggestion regarding the manner in which B. perfringens produces its pathogenic effects, which I would enlarge on as follows:

It has been previously mentioned that, as a routine control against contamination, slope agar cultures were made and incubated aerobically. It was noticed that frequently after 36 hours a fine film could be detected on the surface of the agar which on staining proved to be gram-positive bacilli, the majority of which were longer and finer than when grown anaerobically. If left in the incubator or even in room temperature these cultures often showed fairly luxuriant growth. A thick emulsion in salt solution from two such 52-hour agar cultures failed to produce any gross local lesion when inoculated into the breast muscles of a pigeon, although the 18-hour muscle glucose broth culture to which these agar cultures acted as controls killed a pigeon in a dose less than 0.01 c c in 8 hours.

When these results are viewed in the light shed by the apparently similar effects, local and general, that the inoculation of whole culture and filtrate (toxin) will regularly produce (except the gas infiltration), they emphasize

the conclusion reached by Bull and Pritchett that exotoxin, which the Welch bacillus produces under suitable conditions of growth, by itself can act on the tissues in a manner identical with the action of the whole cultures. It would further appear that the bacilli if injected by themselves and not in combination with exotoxin may prove nonpathogenic whether the bacilli had previously been separated from their exotoxin by centrifuging and washing or grown in such a fashion that toxin was not produced either in vivo or in vitro.

The detailed results obtained by Bull and Pritchett on the influence of different mediums and the period of incubation with regard to the potency of the toxin allowed them to summarize these factors in the statement "that toxicity of the filtrates is inversely proportional to the incubation time calculating from the end of the first day, and that this general relation obtains independently of the nature of the medium. . . . The rapidity of the decrease in toxicity, however, is materially influenced by the percentage of glucose in the medium and the presence of raw muscle. In my hands it became increasingly noticeable that a decrease of the infectivity of the culture began before 24 hours.

When filtration of the culture was desired it was found difficult to get sufficient material from the killed pigeon to inoculate the necessarily large amounts of medium required and on this account a short incubation of from 6-8 hours was tried with culture tubes heavily planted from the bird lesion. Luxuriant growth took place in this time and provided an excellent parent culture for the larger amounts of medium designed for filtration. If the pigeon inoculation was so timed that death took place during the night the intermediate culture tubes could be planted early in the morning and used to inoculate the larger amounts of medium late that afternoon, so that filtration could be started early next day after from 15-18 hours' (or even less) incubation.

By the injection of pigeons with these short incubation cultures it was found that even 6-8 hours' incubation produced a culture of high virulence. This rapid production of toxin was not observed until late in the course of the work and consequently was not as fully investigated as its importance warranted both for practical and academic purposes. Previous to these observations the routine results had continuously led to shortening of the incubation time. It was to some extent on this account that the use of the intermediate culture was tried because when large amounts of medium were inoculated directly from the bird lesion luxuriant growth frequently failed to take place within 18 hours' incubation. Filtration of such insufficiently grown cultures gave a toxin of low potency.

Acting on the suggestion provided by the demonstrated infectivity of 6-8 hour cultures the virulence of the strain was easily maintained (in contrast to the earlier experience) between the time when the horses were bled and the final testing was carried out. By this method one had a cycle of bird subculture and bird incubation with ensuing death in 24 hours.

As noted by other workers, considerable loss of toxicity took place during the process of filtration, so that the time involved was shortened as much as possible by the following technic. The oil covered cultures were passed through a paper mash which freed the fluid from all broken bits of muscle tissue, and if stopped before any of the oil passed through, a fairly clear and oil free fluid was obtained. This was filtered through a medium pore Berkefeld candle and collected in sterile colored glass bottles containing a few cubic centimeters of sterile paraffin oil.

It was tentatively assumed that for immunization purposes the injection of a filtrate of high potency rather than a larger amount of filtrate of lower virulence (though the two amounts might represent the same number of M L D's) would be more likely to produce an antiserum of higher protective strength.

On this conception cultures were not used for filtration unless at least 0.02 c c of the supernatant fluid produced death in a pigeon weighing from 300-350 gm. in 10-12 hours. From the literature, I have gained the impression that this was easily obtained but in my hands I found that considerable care had to be exercised to keep the virulence up to this point, and further, that considerable time would be lost before the virulence could be raised again once this was allowed to sink to such an extent that over 1 c c of the culture had to be injected intramuscularly into the pigeon to cause death.

Standardization of Antiserum

The Bull-Pritchett method of estimating the antitoxic titer of the serum to be tested was essentially the determination of the amount of serum necessary to completely neutralize 1 M L D of toxin. The various serum dilutions and 1 M L D toxin were incubated for one hour and then injected into the breast muscles of the pigeon. Absolute neutralization as shown at the site of inoculation was the criterion by which this was estimated. By this method one was occasionally left in doubt as to which bird showed absolute neutralization, and it seemed advantageous that the determination of this point should not rest on an opinion but on some fact. To realize this, 2 M L D's of toxin were used as the test dose and the smallest amount of serum which would allow the bird to survive 36 hours was determined. The serum-toxin mixtures were incubated for one hour and the smallest amount of serum required to save the life of the bird estimated as 1 antitoxin unit.

Dr. G. W. McCoy of the United States Public Health Service was kind enough to forward to me the regulations contemplated by his department regarding antiserum against B. perfringens. Their method of standardization suggested that 10 M L D's of toxin be used as the test dose against which the serum to be tested should be diluted to at least 1 in 100. The dilution affording protection against this test toxic dose was called one antitoxic unit. Consequently, 1 c c of an undiluted serum fulfilling these requirements represented protection against 1,000 M L D's of toxin. They further proposed to make it essential that 1 c c of an acceptable serum should have an antitoxic value of 10 such antitoxic units. There seemed to be no particular advantage in using 10 M L D's while at the same time it necessitated the injection of five times the amount of toxin-antitoxin mixture. Pigeons of a weight close to 325 gm., or as recommended by Bull and Pritchett, 350 gm., seemed to give the most consistent results. The results obtained with birds weighing 300 gm. were liable to give somewhat erratic results but had frequently of necessity to be used. Under the circumstances the use of two pigeons per dose was an advisable procedure, as suggested by the United States Public Health Service.

Protective Value of Antiserum

The characteristic lesions following the inoculation of animals and birds with cultures of the different anaerobes recovered from gas wound infection have been so fully described by most of those who have worked on gas gangrene that it seems superfluous to add to the descriptions already given. Bull and Pritchett in particular drew attention to the fact that with B. perfringens the causative factor was probably wholly due to the exotoxins and that, except for the gas formation, the toxins[2] and the whole culture produced practically identical results.

[2] Pease, Marshall C., Proc. Soc. Exper. Biol. and Med. 1919, 17, p. 30.

Weinburg and Seguin,[3] in their most exhaustive work, and Sacquepée,[4] in his various publications. have dealt extensively with the pathogenicity of the various anaerobes and the types of lesion that can be produced experimentally by each or by various combinations of the different anaerobes.

For the purpose for which this work was begun it was thought sufficient to disregard this phase in view of the results obtained by Bull and Pritchett after the following experiment (table 1) which was designed roughly to demonstrate whether the antitoxic serum was protective alike to whole culture and to toxin filtrate.

TABLE 1

Results of Experiment to Show Protective Power of Antitoxic Serum for Whole Culture and Toxin Filtrate

Pigeon	Toxin per 100 Gm., c c	Toxin per Pigeon, c c	Whole Culture per Pigeon, c c	Prophylactic Injection Antitoxin 3 Days Before, c c	Therapeutic Injection Antitoxin After 5 Minutes, c c	Result	
						After 4 Hours	Later
839	0.02	...	....	...	...	Sick	Died emaciated 21 days later
9839	0.04	...	....	...	...	Very sick	Survived
838	0.06	...	....	...	. .	Very sick	Died during night
26	0.08	...	....	...	...	Very sick	Died during night
4463	0.1	...	....	...	...	Moribund	
8826	0.2	...	....	...	...	Dead	
834	....	...	0.25	...	...	Dead	
842	....	...	0.15	...	...	Dead	
835	....	... .	0.05	...	...	Dead	
476	....	5.0	....	2.0	...	Alive and well	No effects
479	....	1.0	....	2.0	...	Alive and well	No effects
480	....	...	0.25	2.0	...	Alive and well	No effects
843	....	2.0	....	...	2.0	Alive and well	No effects
840	....	0.5	....	...	2.0	Alive and well	No effects
841	....	...	0.25	...	2.0	Alive and well	No effects

Weinberg and Seguin describe three types of antiserum to B. perfringens, namely: a polyvalent antibacterial serum, a monovalent antibacterial serum and an antitoxic serum. The first was prepared by inoculating the horse with increasing doses of bacilli freed from toxin by washing and then continuing with increasing doses of whole fluid culture. The second was prepared by inoculating with increasing doses of bacilli freed from toxin by washing and for this serum the most virulent strain available was used. They report that with this latter strain the horse developed severe reactions—temperature 39 C., edema of the legs, etc. The third, namely antitoxin, was apparently not actually produced as under this heading it is stated that "the production is dependent on obtaining regularly a potent toxin and that the problem is associated with a number of difficulties."

[3] La Gangrene Gazeuse, 1918.
[4] Presse méd. 1918, 22, p. 197.

The most effective antiserum was the monovalent which appeared to protect against at most, 2 M L D's of toxin in a dilution of 1 : 200.

According to the deductions which my results would lead me to make even this degree of antitoxin production resulted from the presence of toxin which was either carried over with the bacilli or produced by the bacilli after they had been injected into the host, to which latter possibility the severe reactions would lend some support. The particular cultures used would appear to be less virulent than I found it advisable to employ for immunization purposes as the M L D was at the best 0.5 c c.

Certain features which in the literature at my disposal I have not seen commented on, and which would seem to be of sufficient importance to be recorded may be summed up thus:

With any fairly potent toxin according to the size of the dose it would seem possible to produce death in any desired space of time from about 5 minutes to 24 hours, after which it is problematical whether the pigeon will permanently recover with the extrusion of a hard calculus-like mass or continue in a state of emaciation and eventually die in from 6 weeks to 3 months.

Following the inoculation of either whole culture or filtrate, death usually takes place within 12 hours, rarely after 24 hours unless it be the greatly delayed death of several weeks' duration, when emaciation has become marked.

No practical difference has been noted between the deaths due to whole culture and toxins, unless it be in the instances when survival took place, that this usually became apparent in the case of toxin injection sooner than when whole cultures were inoculated.

When death took place, after 5-8 hours, it was always initiated by loss of balance so that if one lightly touched the pigeon it would fall over. This method was noted as a routine and death can be predicted almost to the hour from this sign. The possibility of this being due to a definite fixation of the toxin by certain of the body cells (analogous to the fixation which takes place with tetanus toxin) was not investigated. A certain amount of this assumed fixation, when toxin alone was injected, could be observed with recovery. If, however, this sign became marked death was certain.

The interval between the time of the inoculation and death in the pigeon is rapidly shortened by comparatively slight increase in the dose. Thus, toxin 57, which at 0.12 c c per 100 gm. pigeon caused death within 24 hours, killed in from 5-10 minutes when the dose was increased to 1.2 c c per 100 gm. pigeon. The weights of these two pigeons were 300 and 320 gm., respectively. Intermediate doses caused death between these two extremes of 10 minutes and 24 hours.

IMMUNIZATION OF HORSES

Immunization of two horses against B. perfringens toxin was begun June, 1918, and continued by increasing doses until November when they were bled, seven days after the last injection of toxin. In August, 1918, an attempt was begun to obtain a combined antiperfringens and antitetanus serum by giving two horses, for some time under active immunization against B. tetani toxin, additional injections of increasing amounts of B. perfringens toxin. At first the perfringens toxin was administered rather conservatively for fear of producing too violent reactions. However, as this did not follow the dose was increased and the interval shortened, the highest temperature reaction obtained being 104 F. No lasting local reaction or noticeable edema of the legs occurred throughout the course of injections.

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The two horses on B. perfringens, 2 G and 4 G, received, respectively, a total of 1820 c c and 1760 c c. The two horses 36 and 38 on combined injections received 955 c c and 970 c c, respectively. The toxin varied in potency for the different injections between a M L D of 0.04 c c to 0.1 c c per 100 gm. pigeon. The increase of dose was judged by the M L D potency and not by the total amount in cubic centimeters.

TABLE 2

TOXIN TITRATIONS

Pigeons	Weight in Grams	Toxin per 100 Grams of Pigeon, c c	Total Toxin Injected, c c	Result
A	320	0.05	0.16	Survived
D	290	0.06	0.17	Died during night
F	340	0.07	0.24	Survived
B	300	0.08	0.24	Died during night
E	300	0.09	0.27	Died during night
C	310	0.1	0.31	Died during night

TABLE 3

STANDARDIZATION OF ANTITOXIN

Pigeon	Weight in Grams	Toxin, 2 M L D per 300 gm. Pigeon	Toxin, Actual Dose in c c	Number of Horse (Antitoxin)	Antitoxin per 300 gm. Pigeon	Antitoxin, Actual Dose in c c of	Dilution	Result
31	365	0.39 c c	0.474 c c	2G Conc.	0.0005 c c	0.608 c c of	1:1000	Surv.
27	305	0.39 c c	0.396 c c	2G Conc.	0.0004 c c	0.405 c c of	1:1000	Surv.
33	310	0.39 c c	0.403 c c	2G Conc.	0.0003 c c	0.310 c c of	1:1000	Surv.
32	245	0.39 c c	0.318 c c	2G Conc.	0.0002 c c	·0.163 c c of	1:1000	Surv.
28	275	0.39 c c	0.359 c c	2G Conc.	0.0001 c c	0.093 c c of	1:1000	Surv.
30	345	0.39 c c	0.448 c c	2G Lymph	0.0005 c c	0.575 c c of	1:1000	Surv.
34	385	0.39 c c	0.50 c c	2G Lymph	0.0004 c c	0.513 c c of	1:1000	Surv.
29	395	0.39 c c	0.513 c c	2G Lymph	0.0003 c c	0.395 c c of	1:1000	Surv.
41	285	0.39 c c	0.37 c c	2G Lymph	0.0002 c c	0.190 c c of	1:1000	Surv.
37	295	0.39 c c	0.38 c c	2G Lymph	0.0001 c c	0.098 c c of	1:1000	Surv.
51	250	0.39 c c	0.32 c c	2G Conc.	0.0002 c c	0.83 c c of	1:5000	Surv.
52	270	0.39 c c	0.34 c c	2G Conc.	0.0001 c c	0.45 c c of	1:5000	Surv.
53	270	0.39 c c	0.34 c c	2G Conc.	0.00006 c c	0.29 c c of	1:5000	Surv.
54	280	0.39 c c	0.36 c c	2G Conc.	0.00005 c c	0.23 c c of	1:5000	Surv.
55	260	0.39 c c	0.33 c c	2G Lymph	0.0002 c c	0.866 c c of	1:5000	Surv.
56	260	0.39 c c	0.33 c c	2G Lymph	0.0001 c c	0.43 c c of	1:5000	Surv.
58	280	0.39 c c	0.36 c c	2G Lymph	0.00006 c c	0.308 c c of	1:5000	Surv.
59	250	0.39 c c	0.32 c c	2G Lymph	0.00005 c c	0.208 c c of	1:5000	Surv.
23	300	0.39 c c	0.39 c c	2G Conc.	0.00005 c c	1.0 c c of	1:20000	Surv.
7	360	0.39 c c	0.47 c c	2G Conc.	0.000025 c c	0.6 c c of	1:20000	Surv.
10	290	0.39 c c	0.38 c c	2G Conc.	0.00001 c c	0.19 c c of	1:20000	D. 24 hr.
11	250	0.39 c c	0.32 c c	2G Lymph	0.00005 c c	0.83 c c of	1:20000	D. d. n.
12	250	0.39 c c	0.32 c c	2G Lymph	0.000025 c c	0.42 c c of	1:20000	D. d. n.
13	280	0.39 c c	0.36 c c	2G Lymph	0.00001 c c	0.19 c c of	1:20000	D. d. n.

Pigeon	Weight in gm.	Toxin 136 per 100 gm. Pigeon	Actual Dose of Toxin	Result
3	255	0.05 c c	0.13 c c	Survived
1	250	0.06 c	0.015 c c	Died during night
2	260	0.07 c c	0.18 c c	Died during night

Surv. = survived; D. = died; D. d. n. = died during night.

A trial test of the two horses 2 G and 4 G on August 23, 1919, showed neutralization by the method of Bull and Pritchett, for the 2 G serum in a dilution of 1:600 to 1:800, and for the 4 G serum in a dilution of 1:600.

The protocol for standardization of antitoxin suggested by the United States Public Health Service corrected the actual dose of antitoxin as well as toxin according to the weight of the bird. This seemed a desirable modification and was thereafter adopted.

The final standardization of the antiserum was postponed until both serum, concentrated by the method of Banzhaff, and Lymph could be contrasted.

In the standardization of toxin slight irregularities were occasionally encountered, so that one might be left in some doubt regarding the exact dose which should be considered the true M L D for the subsequent standardization of the antiserum. The use of a 2 M L D amount as the test dose has a practical value in that any error, made in the estimated M L D, will be exposed by the additional controls made in the standardization of antitoxin experiment and can be taken into account. The standardization of the antiserum was proceeded with as soon as the M L D of the test toxin was obtained on account of the possible deterioration of the toxin.

Table 2 shows the results of the titration of toxin, and table 3, the method adopted for routine for standardization of antitoxin.

Table 4 shows the results obtained for eight samples. For the data on the antitetanic serum I am indebted to the Antitoxin Division of the Connaught Laboratories.

TABLE 4

Experiment on Four Horses with Perfringens Toxin Alone
and Combined with Tetanus Toxin

Antitoxin	Smallest Amount of Antitoxin (per 300 Gm. Pigeon) Affording Protection Against 2 M L D's Toxin	Number of Antitoxin Units per c c (perfringens)	Number of Antitoxin Units per c c (tetanus)
2G concentrated	0.000025 c c	40,000	
2G lymph	0.000066 c c	15,000	
4G concentrated	0.000025 c c	40,000	
4G lymph	0.00005 c c	20,000	
36 concentrated	0.0001 c c	10,000	700
36 lymph	0.0002 c c	5,000	150
38 concentrated	0.000066 c c	15,000	700
38 lymph	Less than 0.0005 c c	2,000 (?)	150

DISCUSSION

With the cessation of the war the urgent necessity for the production of antiserum against gas gangrene has largely subsided. However, a sufficient number of cases are encountered in civil practice to warrant further work both for practical and scientific purposes.

The large amount of routine and experimental work carried on especially during the latter years of the war has given us a fairly well accepted identification of the bacterial flora, anaerobic and aerobic, encountered in gas gangrene following war wounds, has separated

those essentially important etiologically, and has shown that experimentally the different infections can be prevented by their specific antitoxins, and can even be successfully treated after infection, if the interval, before treatment is begun, is not too long delayed.

Van Beuren [5] in a clinical review states that "while serum therapy promises much for the future it has not had much opportunity for performance in the past, or if it has had the reports are not yet available."

Weinberg and Seguin [3] give full details in 30 controlled cases which received specific treatment. Of these, 11 died and 19 recovered. The 11 instances of mortality are divided into 3 groups as follows:

1. The antiserum used did not correspond to the anaerobe found by bacteriologic investigation in 3 cases.

2. The proper specific serum was employed but treatment was begun too late in 5 cases.

·3. The proper specific serum was employed and apparently had some effect on its specific infection but death followed from other complications in 3 cases.

The 3 antiserums used were antiperfringens, anti-oedematiens, and antiseptique. The antitoxic titer is not given, but the amounts of serum injected varied from 20 to 50 c c daily or oftener. The inoculations were given both subcutaneously about the wound and intravenously.

If one accepts B. bellonensis (B. de l'oedeme gazeus malin) of Sacquepée as identical with the B. oedematiens, as is admitted probably by both Weinberg and the Medical Research [6] Anaerobic Committee, practically all observers are in accord regarding the etiologic rôle of B. perfringens, B. oedematiens and V. septique, though Sacquepée would appear to question the essentially etiologic importance of B. perfringens chiefly on these grounds:

1. Cultures and films taken from guinea-pigs inoculated with a mixture of V. septique and B. perfringens showed that B. perfringens had grown much more luxuriously than V. septique, both in the host and the cultures; however, when inoculated separately, V. septique had been demonstrated to be much more virulent.

2. He considered these observations to be a probable explanation of the greater frequency with which B. perfringens was recovered from war wounds, and that though important, its pathogenicity came into play as a secondary invader.

[5] Jour. Am. Med. Assn., 1919, 73, p. 239.

[6] Medical Research Committee Reports, Classification and study of the anaerobic bacteria of war wounds. Series No 12, 1917.

3. Pathogenicity was frequently lessened and variable as noted experimentally with B. perfringens in contrast with V. septique or B. oedematiens.

Regarding the frequency with which only one strain could be identified, Weinberg reports the following results for the 12 cases in which monoinfection was found in his series: B. perfringens 9 times, B. oedematiens twice, and B. fallax once.

Judging from the extreme ease and rapidity with which the two strains of B. perfringens, 617 D and B 2, would fail to show pathogenicity because the production of exotoxin was inhibited either in vivo and in vitro, these observations are not to my conception contradictory to the importance of B. perfringens etiologically, but support the idea that the environment, be it due to lacerated tissue,[7] other micro-organisms or any favorable cause, must be such as to encourage the production of exotoxin before B. perfringens exerts its pathogenic effect.

In this connection, an interesting observation was made by Weinberg and Seguin regarding the action of the filtrate of B. sporogenes on the toxin of B. oedematiens, V. septique and B. perfringens. They were able to demonstrate that while the toxin of B. perfringens was unaffected by incubation with the filtrate of B. sporogenes, the toxins of both B. oedematiens and V. septique were materially attenuated.

The National Medical Research Anaerobe Committee report that in many cases B. perfringens apparently existed as a saphrophyte without etiologic importance. In this same report they come to the conclusion that only two types of anaerobes, namely, B. tetanus and B. botulinus, can be considered to be truly toxogenic. They consider it "doubtful if the products of the other anaerobes can be regarded as toxins in the true sense of the word as their injection is usually followed by immediate toxic symptoms."

Regarding this, I think one might say that our conception of toxin is based on the side chain theory, and that the two chief essentials which must be incorporated into a definition of the word toxin in the restricted bacteriologic sense are:

1. It is a specific poison secreted by a pathogenic micro-organism (specific poison being defined as the substance giving rise to the chief pathologic effects which taken together constitute the disease—Dean.[8]

[7] Vincent et Stodel: Compt. Rend. Acad. Sc., 1917, 164, p. 870.

[8] The Bacteriology of Diphtheria, Nuttal and Smith 1908.

2. This soluble substance secreted in vivo or in vitro is capable by its action on the animal body of the production of a specific antibody.

Other criteria by which toxins may be characterized are: unknown chemical structure, lability, effectiveness of minute dosage, production of pathogenic effects by most of the toxins only after a latent or incubation period.

Dean places 2 and the last criterion in this order as necessary characteristics of a toxin.

B. perfringens filtrate conforms to the definition as suggested and has all the characteristics except that it does not produce pathogenic effects only after a latent period. On this account it would appear to be able to act in a manner different from the toxins produced, for example by B. diphtheriae or B. tetani.

As to which particular manifestations of action and characteristics a poison produced by the growth of a micro-organism must have before it is to be regarded as a toxin in the true bacteriologic sense, is to my mind relatively unimportant to a more accurate understanding of the method of action. It seems an essential conception that the manner in which action is produced must be entirely different if for one there has to be a period of latency, whereas for the other no such period exists, rapidity of action apparently being dependent on the size of the dose, or otherwise expressed on the number of M L D's injected.

For both diphtheria and tetanus toxin the latency period can to some extent be curtailed but not abolished even if many hundred or thousand multiples of the M L D be injected. In the case of diphtheria toxin Dean states this may be reduced to from 8-12 hours.

The same author also states that "the bacilli obtained from cases of diphtheria have as a rule a toxicity which can be expressed by stating that from 0.1 c c to 0.02 c c of the filtrate of a ten days old culture injected subcutaneously into a guinea-pig of 250 gm. weight kills the animal within five days." This size dose is comparable to that of the filtrate of a suitable B. perfringens culture.

No attempt was made to investigate the mechanism by which death was produced by the filtrate of B. perfringens cultures, although such an attempt might throw light not only on B. perfringens toxin but also on the more thoroughly investigated toxins of B. diphtheriae and B. tetani. A classification of toxins based on manner of action would be, I think, a more likely outcome than that the filtrate of B. perfringens cultures would be excluded from the group of true bacteriologic toxins.

Weinberg and Seguin place five anaerobes in the following order of frequency and importance etiologically: (1) B. perfringens, (2) B.

oedematiens, (3) B. sporogenes, (4) B. fallax, and (5) V. septique. B. sporogenes would appear to be the most dangerous of those anaerobes (or aerobes) which are the cause of the putrid odor so frequently noticed in war wounds. V. Septique while not so frequently encountered, has always shown marked pathogenicity, so that antiserum to 1, 2, and 5 at least is desirable and if available should allow specific treatment in the great majority of cases. The antiserum prepared by these authors to both B. oedematiens and V. septique is strictly an antitoxin and was eventually obtained in high potency—about 50,000 or more antitoxic units to 1 c c. Thus one can state that as far as these three strains—B. perfrigens, B. oedematiens and V. septique— are concerned, their pathogenicity is due to an exotoxin against which with suitable strains it is possible to immunize horses and obtain potent antitoxins. It would appear that each antiserum was specific for all members of that strain, but that antitoxins for B. perfringens would not be effective against V. septique, B. oedematiens or vice versa.

As has been shown, it is possible to produce a combined antitetanus and antiperfringens serum from one horse. That anti-B. oedematiens and anti-V. septique could also be combined is possible, though on this 1 have not noticed any references in the literature at my disposal.

Experimentally all the results point to the great value of the prophylactic use of antiserum specific to the infecting anaerobe, and to the urgency of the earliest institution possible of specific treatment once the disease is recognized. Weinberg and Seguin's controlled cases are encouraging results for the use of specific treatment provided there is as little delay as possible before it is started, and care is taken to determine which anaerobe (or anaerobes) are present.

Judging from the data supplied by the studies of the anaerobes found to be etiologically important in war wounds, it would appear advisable in civilian cases to inject all three antitoxins as early as possible, and to determine the anaerobe (or anaerobes) present in the particular case so that the administration of the appropriate antitoxins could then be pushed.

Weinberg's antitoxin to V. septique and B. oedematiens would appear to contain at the least 10 antitoxic units per cubic centimeter of serum as defined by the United States Public Health Service. One could thus administer daily a minimum of 250 antitoxic units to all three anaerobes in a total of 75 c c of serum until the etiologic agents for the particular case were determined.

As pointed out by Van Beuren, the specific treatment should be regarded as an adjunct to full and proper surgical measures, and should not in any way supplant these surgical steps.